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Original Article

Transformation of tobacco plants by Yali PPO-GFP fusion gene and observation of subcellular localization

Jing Qi[1], Gui-Qin Li[1], Zhen Dong[2], Wei Zhou[1]

[1]*College of Biology Science and Engineering, Hebei University of Economics and Business, Shijiazhuang 050061, Hebei, China;* [2]*Hebei Women's Vocational College, Shijiazhuang 050091, Hebei, China*

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Abstract: To explore the subcellular localization of Polyphenol oxidase (PPO) from Pyrus bretschneideri, the 1779 bp cDNA of PPO gene excluding the termination codon TAA was cloned and fused with GFP to construct a binary vector pBI121-PPO-GFP. Then, the binary vector was transformed into Nicotiana tabacum by the tumefanciens-mediated method. Using confocal laser scanning microscopy, green fluorescent signals were localized in chloroplasts of the transformed Nicotiana tabacum cell, suggesting that the Polyphenol oxidase from Pyrus bretschneideri was a chloroplast protein.

Keywords: Yali, PPO, GFP, gene fusion, transformation, subcellular localization

Introduction

Numerous studies have confirmed that browning in fruits and vegetables is mainly caused by enzymatic reaction mediated by oxidoreductase, oxidizing polyphenols to dark brown quinones leading to browning. Polyphenol oxidase (PPO) is the main enzyme catalyzing the oxidation-reduction reaction. Browning can be inhibited by reducing enzyme content or inhibiting enzyme activity, commonly being used to retard browning on fruits and vegetables. Because of the important role of polyphenol oxidase in browning, polyphenol oxidase has attracted the attention of many scientists. Further study on PPO will help solve the browning problem on fruits and vegetables fundamentally.

Previous studies have confirmed that polyphenol oxidase is a plastid enzyme, encoded by nuclear genes and synthesized in the cytoplasm before transferred to other regions. Chemical and immunochemical analysis of cells showed that polyphenol oxidase mainly presents in photosynthetic tissues of normal cells (such as vesicles thylakoids) and non-photosynthetic plastids (such as amyloplast in potato tubers) [9, 13, 15]. The tissues without plastid have no polyphenol oxidase, but the plant tissues containing plastid probably have PPO, and the reason is not clear so far. Although the polyphenol oxidase widely exists in the plastid of plant cells, polyphenol oxidase for different sources presents in various types of plastids [3, 14]. Currently, localization of polyphenol oxidase in cells is still gaps in research. Although previous study reported PPO gene of Yali obtained by cloning localized in the cell thylakoids through bioinformatics analysis [11], it is still short of strong experimental results to support it. Therefore, as part of the study of gene function, we intended to verify it by fusing PPO with GFP for genetic co-transformation and subcellular localization of Yali polyphenol oxidase, in order to lay the foundation for further study of genes encoding the protein.

Materials and methods

Materials

Plants: Pyrus bretschneideri 'Yali' pear trees were planted in Shijiazhuang Institute of fruit trees, Hebei Academy of Agriculture and Forestry Sciences, with routine management. Nicotiana tabacum Samsums were cultivated by the laboratory: 22e, 16 h light/8 h dark, luminous flux density of 110 $L/m^{-2}/s^{-1}$, humidity of about 30%.

Reagents: E. coli strain DH5α, cloning vector pUCm-T Vector, small amount of UNIQ-10 column plasmid extraction kit and DNA gel extraction kit were purchased from Shanghai Sangon biotech Company; Oligo (dT) 15, RTase M-MLV, Taq enzyme, dNTP, restriction endonucleases, T4 ligase and DNA Marker were purchased from TAKARA company; Agrobacterium strain EHA105 and binary expression vector GFP-pBI121 containing the GFP gene was provided by our laboratory; other conventional chemical reagents were all AR.

Methods

Construction of fluorescent expression vector: Total RNA of ripe pear was extracted referring to the improved CTAB method [4] and reverse transcribed into cDNA. A pair of primers was designed according to the CDS region of obtained full-length cDNA sequences of pear PPO [11] (upstream primer: 5'-TGACGTCTC-TTTCACCTCCGGTAGTCA-3', downstream primer: 5'-AGAAGCAAACTCAATCTTGATAC-3'). Complete amino acid coding sequence excluding stop codon TAA was amplified using Yali cDNA as a template in a system of 50 μL, containing pear DNA 50 ng, 10 × PCR Buffer 5 μL, rTaq (TAKARA) 0.5 μL, each 30 ng upstream and downstream primer, dNTP with a final concentration of 0.25 mmol•L-1. PCR reactions were performed using T Gradient PCR instrument (Biometra, Germany). The amplification program as follows: denaturation at 94°C for 30 s, annealing at 70°C for 30 s, extension at 72°C for 2 min, total 35 cycles.

After PCR amplification, the amplified product was separated and recovered by agarose gel electrophoresis. E.coli DH 5α competent cells were transformed by the complex of PCR product and pUCm-T Vector before being sequenced by Shanghai Sangon biotech. According to the assay results, target sequence fragment was selected and inserted in the recombinant plasmid in cloning sites of pUCm-T Vector and fluorescent expression vectors GFP-pBI121 by XbaI/SacI double digestion. After digestion reaction, PPO gene fragment and GFP-pBI121 linear vector were recovered by electrophoresis, and the two fragments were ligated to construct PPO-GFP fluorescence expression vector. After verification by sequencing, PPO-GFP fluorescence expression vector was used to transform Agrobacterium strain EHA105 for tobacco transformation by freeze-thaw method [1].

Tobacco transformation by fluorescence expression vector mediated by Agrobacterium tumefaciens LBA4404PPO-GFP: 1 to 2 months-old Nicotiana tabacum Samsums were selected. Edges and main vein of delicate leaves were cut into small pieces (about 0.5 cm × 0.5 cm) as the starting explants for transformation.

Infection and transformation of tobacco were performed as the leaf disc method [8]. Firstly, Agrobacterium engineering strain EHA105 was picked with inoculation needle under sterile conditions, and streaked on the YEB plate containing 50 mg/mL Kan, Rif and Str respectively to obtain single colonies at 28°C in dark by inverted culture. Single colonies were picked on the transformation plates, and inoculated into 1 mL YEB liquid medium containing the same antibiotics, at 220 rpm and 28°C, shaking overnight. 1 ml of the above culture was added to 50 ml YEB liquid medium containing the same antibiotic, and incubated and shaked at 220 rpm and 28°C for about 14 h, obtaining 0.6 of OD600; The culture was centrifuged at 5000 rpm for 15 min in room temperature; the supernatant was discarded and cells were suspended in MS liquid medium, diluted 10-fold for use. Then the prepared tobacco leaf disc was placed in the bacterial suspension for 5 min with gentle shaking and incubated with the co-culture medium (MS + 1 mg/L 6-BA) at 26°C in dark for 3 d; screening and differentiation were performed in the screening medium (MS + 1 mg/L 6-BA + 500 mg/LCef + 30 mg/LKan); subculture was performed once every two weeks, and after three or four weeks, shoots had differentiated from the edge of the blade; resistant shoots longer than 1 cm were cut and inserted in rooting medium (1/2 MS + 500 mg/L Cef + 30 mg/L Kan) for rooting. After 5 to 8 taproot forming, sterile seedlings were cultured at 25°C for 2 d with the lid opening before being transplanted into the soil by conventional management in the greenhouse.

PCR detection of transgenic tobacco: According to the GFP gene sequence published in Genbank, a pair of primers was designed with the upstream primer: 5'-GTTGAATTAGATGGTG-ATAAT-3' and downstream primer: 5'-ATAAC-CTTCGGGCATGGCACTC-3'. Referring to the CTAB method [1], DNA of transgenic tobacco

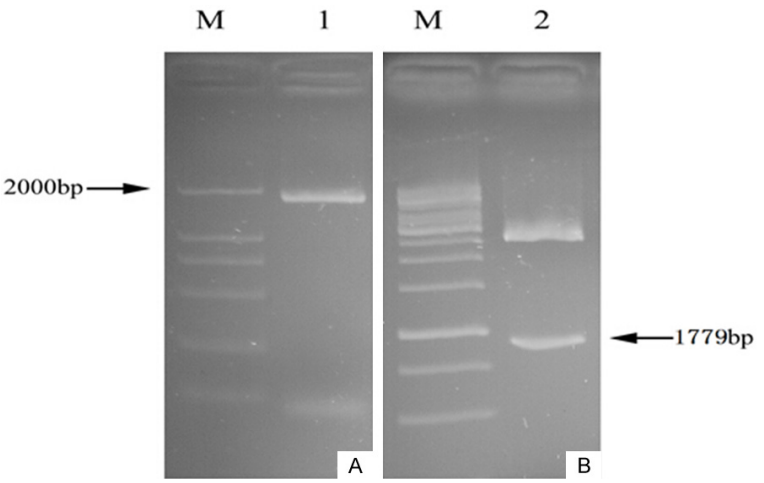

Figure 1. PCR implification of *PPO* and identification of cloning vector by endonuclease digest. Note: (A) PCR amplification products of *PPO*; Lane M: 1 Kb DNA marker (2000 bp, 1000 bp, 750 bp, 500 bp, 250 bp, 100 bp); Lane 1: Product of PCR implification. (B) Product cut by *EcoR* I/*BamH* I. Lane M, DNA marker; Lane 2: Product by endonuclease digest.

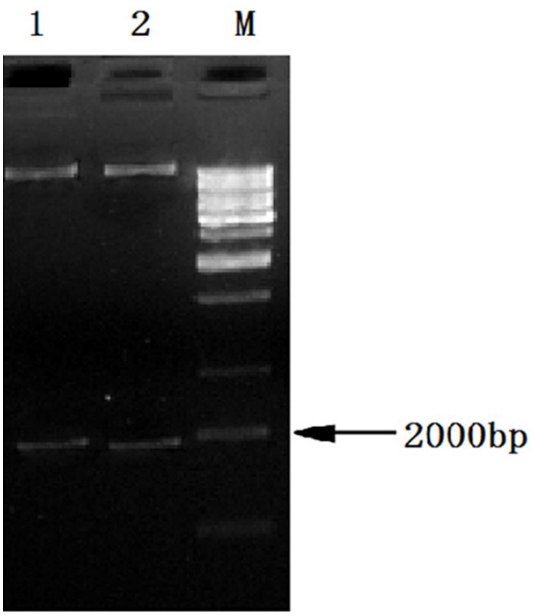

Figure 2. Identification of pBI121-PPO-GFP by restriction enzyme digestion. Note: Lane 1 & 2: Recombinant plasmid doubledigest by Sac I and Xba I; M: 1 kb DNA Ladder.

was extracted, and amplification was carried out using it as a template. Amplification system was consistent with 1.2.1; amplification program as follows: denaturation at 94°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 90 s, a total of 35 cycles. After PCR amplification, the amplified product was separated by 1% agarose gel electrophoresis, detected and photographed by UV.

Laser scanning confocal microscope detection of transgenic tobacco: Leaves of transgenic tobacco confirmed by PCR were observed using a laser scanning confocal microscope (LSM5 Pascal, Zeiss) to detect the expression of GFP in mesophyll cells. 40 × oil immersion was used in observation with an excitation wavelength of 488 nm, bandpass BP of 505~530 nm and longpass LP of 560 nm.

Results and analysis

Construction of fluorescent expression vector

A pair of primers was designed according to the full-length cDNA sequences of pear PPO [11]. Complete amino acid coding sequence excluding stop codon TAA was amplified by RT-PCR taking Yali mDNA as a template. The results were shown in **Figure 1A**. After being recovered, the target fragment was linked with pUCm-T Vector to transform E.coli DH 5α competent cells, which was accurately identified by blue-white screening and plasmid restriction analysis (**Figure 1B**) before being sequenced by Shanghai Sangon biotech. The sequencing results showed that the sequence of 1779 bp was consistent with that of the known pear polyphenol oxidase gene, without base mutation, which can be used to construct the plant expression vector.

There were two insert modes (forward and reverse) to link amplified gene fragment of Pear polyphenol oxidase and pUCm-T Vector. Reverse plasmid of pUCm-T Vector and Pear polyphenol oxidase verified by sequencing was selected. XbaI/SacI double digestion was performed and target fragment was recycled by electrophoresis, and targeted with the same digested pBI121 linear vector containing GFP gene. After connecting, products were transformed into E.coli DH 5α; kanamycin (Kan)-resistant monoclines were picked for further propagation. After extracting the plasmid, polyphenol oxidase gene fragment was successfully inserted into an expression vector confirmed by XbaI/SacI double digestion (**Figure 2**). Sequencing results showed that the target fragment showed a forward arrangement between

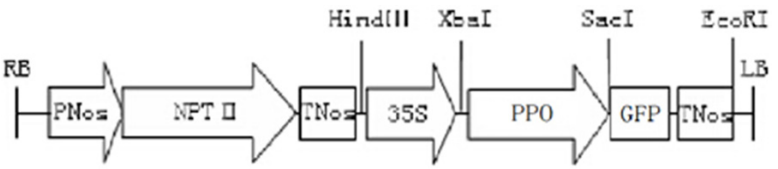

Figure 3. Diagram of antisense express vector of pBI121-PPO-GFP.

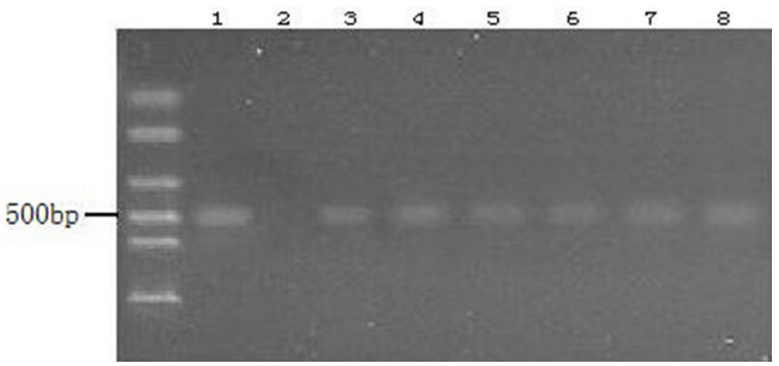

Figure 4. PCR analysis of transgenic tobacco plants. Note: Line 1 is positive control; line 2 is negative control; line 3~8 is transgenic tobacco plants.

35S promoter and GFP gene, and PPO-GFP fusion gene transcription was regulated by the 35S son. Fluorescence spectra of the constructed expression vector were shown in **Figure 3**.

Transgenic plants

The constructed plasmid pBI121-PPO-GFP and pBI121 plasmid (pBI121-GFP) only containing GFP gene cells were transformed into Agrobacterium EHA105 with electroporation, referring to the leaf disc method [11] to transform tobacco. The tobacco leaves, co-cultured with two Agrobacterium Engineering strains for 3 days respectively, were inoculated on selective medium inducing culture, sub cultured every two weeks. After six weeks, well growing shoots were inoculated into the rooting medium containing kanamycin for continue incubation; root differentiated in about three weeks; the developed roots were transplanted to nutritional bowl and eventually 77 strains of pBI121-PPO-GFP tobacco and 69 strains of pBI121-GFP tobacco were obtained, with normal phenotype.

PCR detection of transgenic plants

After kanamycin screening for tobacco plants, 10 plants were randomly selected to extract its DNA. It was a template for PCR amplification according to the designed GFP gene primers. Electrophoresis results show that in transgenic PPO-GFP genes in tobacco plants, the PCR test results were positive, while in the GFP transgenic gene tobacco plants only eight amplified the target fragment, with 2 showing a false positive (**Figure 4** show part of the electrophoresis results).

The expression of GFP reporter gene

Laser scanning confocal microscopy was used to observe the expression of GFP in leaves in positive PCR testing tobacco plants. Results were shown in the 488 nm excitation light. Chlorophyll autofluorescence (red) appeared in wild-type tobacco at wavelength 560 nm. There was no signal at wavelength 505~530 nm which was its luminous band (**Figure 5A-C**). At wavelength 505~530 nm band in pBI121-GFP transgenic plants, GFP fluorescence (green) in the cytoplasm dispersed like distribution (**Figure 5D-F**). In pBI121-PPO-GFP transgenic plants, GFP fluorescence signal and chlorophyll autofluorescence signal co-localized in the chloroplast (**Figure 5G-I**). The results showed that pear polyphenol oxidase gene, which was obtained in this study, encoded chloroplast proteins in mature peptide. Thus, this protein is nuclear genes encoded chloroplast protein.

Discussion

The location of protein in organelles plays an important role in its identified function and involved metabolic pathways. The location of Polyphenol oxidase in organelle is complex. It was found in chloroplasts [10], mitochondria [7], peroxisomes and micro-body [12] in other plants. Sommer et al. and Smeekens considered it mainly located in the thylakoid, while Min Wang and Zheng Shi Zhang found that it mainly located in the mitochondria in water hyacinth. Ju Zhiguo also found that the activity of polyphenol oxidase was highest in mitochon-

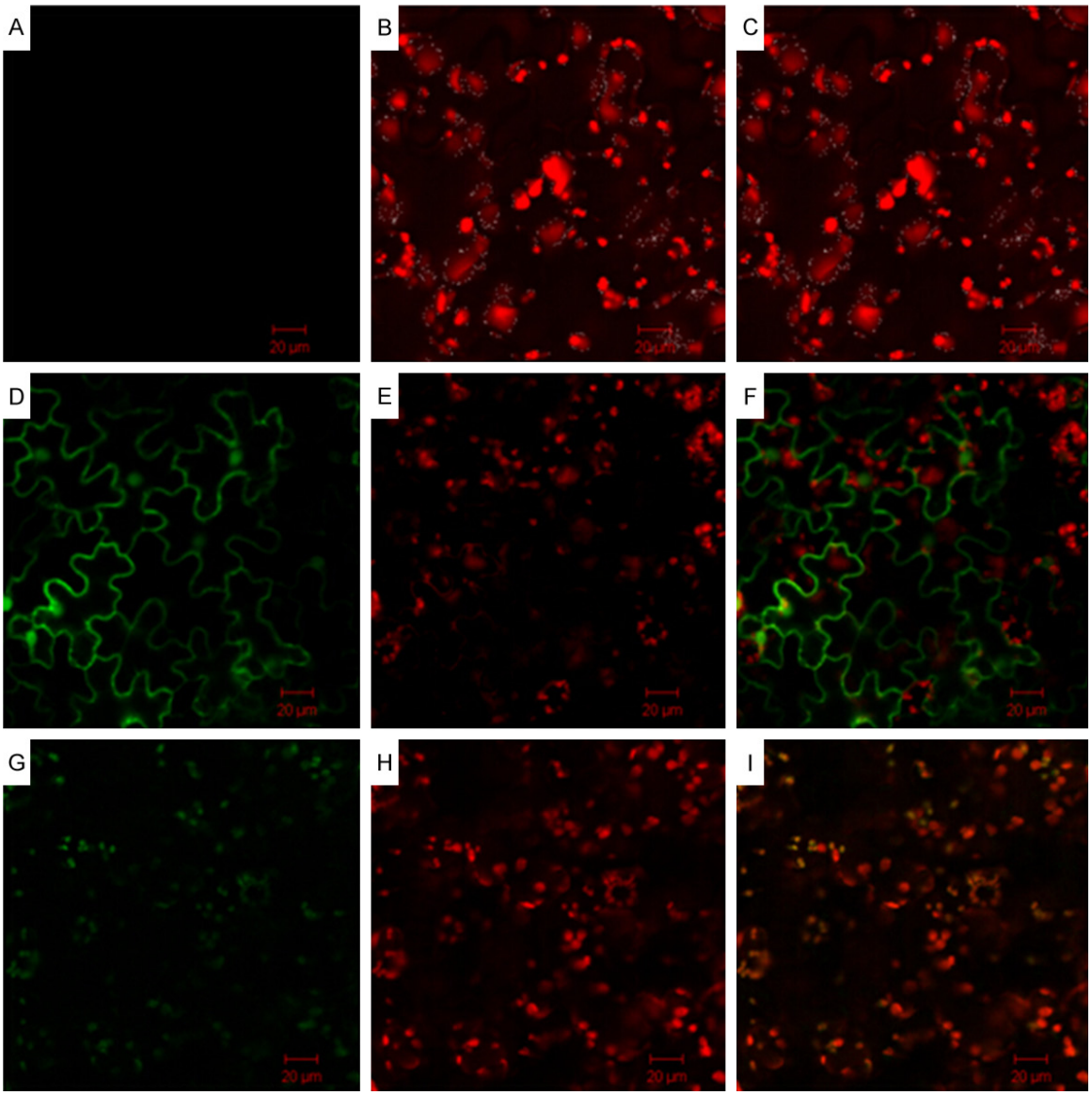

Figure 5. The fluorescence detection in the transgenic tobacco cells. Note: A-C: Wild type tobacco fluorescent detection; D-F: Transgenic plant pBI121-GFP fluorescent detection; G-I: Transgenic plants pBI121-PPO-GFP fluorescent detection; A, D, G are GFP fluorescence; B, E, H are Chlorophyll autofluorescence; C, F I are overlapping. Note: scale bars are 10 μm.

dria of subcellular particles in Laiyang pear. These studies have reported to us a clear message that the polyphenol oxidase from different plant seems to have its own unique cell positioning. Therefore it is difficult to estimate the location organelles of polyphenol oxidase in pear based on previous studies. Nevertheless, in the previous study, we have done lots of bioinformatics analysis based on others' study around the polyphenol oxidase gene in pear [11]. The results showed that the structural characteristics of polyphenol oxidase enzyme in pear were consistent with the one located in chloroplasts in majority of other plants. Therefore, in the previous study, we speculate polyphenol oxidase is likely localized in the chloroplast in pear. Based on this inference, we did not do immunofluorescence handling in other organelles in this study. We have achieved the cellular localization analysis of polyphenol oxidase in pear by auto fluorescence of chloroplast. Therefore in the absence of research background, the detailed bioinformatics analysis is necessary for the localization of the cel-

lular protein, which will effectively save the workload of research workers.

In the field of localization of chloroplast by using GFP reporter gene research in the field of chloroplast protein localization, the predicted transit peptide of target protein is fused with GFP to achieve cellular localization in most reported studies. In this study, we have found that polyphenol oxidase gene of pear deduced that the N-terminal of 87 to 89 region of amino acid sequence comprise a cleavage site of Ala-X-Ala, and N-terminal of 1~89 amino acid sequence region has a large number of hydrophilic amino acid residues. More than half of C-terminal amino acid residues are hydrophobic residues, which was consistent with most of the reported characteristics of polyphenol oxidase transit peptide [5]. However, considering the complex positioning way of polyphenol oxidase in plants, we still use the RT-PCR cloned CDS sequence, which is fused with GFP gene, to achieve cellular localization of polyphenol oxidase in pear conservatively. Although the fused protein location method is more convincing, it failed to confirm the boot peptide sequence. Therefore, further validation of polyphenol oxidase transit peptide sequence in pear will clarify the structure and composition characteristics of polyphenol oxidase after processing of ripe pear. It becomes a priority for us to make a deeply understand of polyphenol oxidase.

In this study, we have succeeded in transforming fluorescence expression vectors into tobacco by using agrobacterium tumefaciens and ultimately achieved pear polyphenol oxidase cellular localization of tobacco. In theory, the expression vectors, which was transformed into pear cell fluorescence, is more convincing by the detection of microscopy. However, in actual operation, we found that, compared with tobacco, the volume of pear cell is too small. It is difficult to locate a light source for GFP. Therefore we can only choose the model plant tobacco to carry out the genetic transformation. Although this is an indirect authentication method at the molecular level, considering the use of cellular localization of GFP reporter gene to achieve protein model plants have become a common approach in the field of protein [2, 6]. And it has a solid theoretical foundation. Therefore, we believe that cellular localization of polyphenol oxidase in pear in this experiment show the same result, and it is located in the chloroplast.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jing Qi, Hebei University of Economics and Business, XueFu Road Number 47, Shijiazhuang, Hebei Province, China. Tel: +86-3118-7657599; Fax: +86-3118-7657599; E-mail: qj2013qj@163.com

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